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Interleukin-3 protects mice from acute herpes simplex virus infection

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SUMMARY

Evidence presented here from kinetic studies of interleukin-3 (IL-3) production by spleen cells from adult mice infected subcutaneously with HSV-1 and stimulated with virus antigen *in vitro* shows that high levels of IL-3 were produced at the onset of the animal's recovery from the disease state. Injections of anti-IL-3 antibody into HSV-1-infected mice resulted in exacerbation of the disease. Primary mouse embryonic head cells grown in the presence of murine IL-3, when infected with HSV-1, showed a 1000-fold decrease in virus titre compared with untreated control cells. This inhibiting effect was reversed by anti-IL-3 and anti-IFN-α, β and γ antibodies. These data suggest that IL-3 plays a host-protective role against HSV infection and it does so probably by inducing brain cells to produce interferons which then inhibit virus replication.

INTRODUCTION

In both humans and mice herpes simplex virus (HSV), a neurotropic virus, can cause acute viral encephalitis leading to death. However, it is unclear how much of the resultant neuropathology is due to viral cytopathy or to the ensuing immune-mediated disease or a combination of both. The protective effect of T-cell responses against HSV-1 infection has been extensively studied, especially with respect to the role of $CD4^+$ (Chan, Lukic & Liew, 1985; Nash *et al.*, 1987) and $CD8^+$ cells (Nash, Field & Quartery-Papafio, 1980; Nagfuchi *et al.*, 1982; Larsen, Russell & Rouse, 1983; Nash & Gell, 1983; Sethi, Omata & Schneweis, 1983). Macrophages are also generally recognized to play an important role in determining the outcome of a herpetic infection. The difference in susceptibility of different age groups or inbred strains of mice to intraperitoneal infection of HSV correlated with a difference in the ability of macrophages to restrict HSV replication and therefore virus dissemination (Johnson, 1964; Hirsch, Zisman & Allison, 1970; Zisman, Hirsch & Allison, 1970; Stevens & Cook, 1971; Domke *et al.*, 1985). However, less is known of the effect of immune cells on HSV-1-induced neuropathology. Infiltration of lymphocytes, accumulation of macrophages and activation of astrocytes (Adams, 1983; Traugott, Scheinberg & Raine, 1985; Hofman, Von Hanwher & Dinarello, 1986) are constant features of the demyelination seen in multiple sclerosis and certain types of virus-induced encephalitis. While the precise role of these cells in the pathology of HSV-1-induced encephalitis is at present unclear, it is possible that this may be mediated via lymphokines.

Several cytokines have been implicated in determining the outcome of a HSV infection. These include interferon (IFN) (α, β, γ) and interleukin-2 (IL-2) (Domke *et al.*, 1985; Engler *et al.*, 1981; Kohl *et al.*, 1989). Considering the importance of cellular immunity in HSV infection, cytokines that regulate the haemopoiesis of bone marrow stem cells may be as crucial. Among the haemopoietic growth factors, interleukin-3 (IL-3) exhibits pleiotropic effects and can stimulate the proliferation and differentiation of pluripotent progenitor cells that are common to both the lymphoid and hemopoietic lineages (Burgess *et al.*, 1980; Clark-Lewis & Schrader, 1981; Yung *et al.*, 1981; Dexter, 1984). In addition to this, IL-3 can modulate colony-stimulating factor (CSF-1) receptors, thereby affecting proliferation of more mature blood monocytes and tissue-derived macrophages. Pertinent to our interest in HSV-induced encephalitis, is the demonstration that multi-CSF, or IL-3, is a mitogen for amoeboid microglia, the intrinsic brain macrophages (Frei *et al.*, 1986; Giulian & Ingeman, 1988).

The questions we have addressed here are whether IL-3 can protect against HSV-1-induced encephalitis and, if so, by what mechanism. We report here that high levels of IL-3 were produced by spleen and lymph node cells from mice acutely infected with HSV and stimulated *in vitro* with virus antigen, at the onset of the animals recovery from the disease state. Monoclonal anti-IL-3 antibody, when injected into HSV-1-infected mice, can exacerbate the disease. Furthermore, highly purified murine IL-3 markedly inhibited HSV-1 replication in primary mouse embryonic head cell cultures. This inhibition is reversible by anti-IL-3 and anti-IFN-α, β and γ antibodies.

Abbreviations: DLN, draining lymph nodes; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PB, polymyxin B.

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MATERIALS AND METHODS

Mice

Young adult male CBA mice aged 7–11 weeks, and 17-day-old CBA embryos, were bred at The Wellcome Research Laboratories, Beckenham, Kent, U.K.

Cells

WEHI-3B cells were maintained in RPMI-1640 containing 10% foetal calf serum (FCS) and 5×10^{-5} M 2-ME (Ziltener *et al.*, 1988). The IL-3-dependent line 32DCL was also grown in the same medium but supplemented with WEHI-3B culture supernatant which was prepared from an overnight culture of 5×10^6 cells/ml of serum-free RPMI-1640.

Virus

As previously described (Chan, 1989), stocks of HSV type 1 Krueger strain were prepared from BHK-21/C13 cells, and virus infectivity was determined by a plaque assay using Vero cells.

Antibodies

The anti-IL-3 hybrid cell line 2E11 (IgG1k) was produced and characterized as previously described (Ziltener *et al.*, 1988). The control of anti-Rubella virus monoclonal antibody WRU (IgG1k) was obtained from Wellcome Biotech Ltd, U.K. A polyclonal sheep anti-IL-3 anti-serum and normal sheep [illegible] were also used. Immunoglobulin was precipitated with 45% ammonium sulphate and dialysed extensively against phosphate-buffered saline (PBS) before administration to mice. The antibodies were adjusted to 1 mg/ml and each mouse was injected i.p. 400 μg 4 hr before virus infection and on Days 1, 3 and 5 post-infection. 2E11 was used in [illegible]B/c mice against synthetic murine IL-3 peptides. It binds to native mouse IL-3 and, when administered *in vivo*, abrogates the increase in splenic mast cells in mice bearing s.c. the IL-3-producing tumour WEHI-3B (Ziltener *et al.*, 1988). The sheep anti-serum can neutralize 1 unit of T-cell-derived IL-3 at 1/50 dilution when assayed against an IL-3-dependent cell line, FDCP1, but has no effect on granulocyte–macrophage colony-stimulating (GM-CSF) factor at the same concentration. Rabbit anti-mouse IFN-α, β and rat monoclonal anti-gamma, were obtained from Lee Biomolecular (San Diego, CA).

Infection of mice

For studies on the effect of anti-IL-3 *in vivo*, male CBA mice aged 7 weeks were infected with $5 \times LD_{50}$ HSV-1 (2×10^6 PFU/mouse) s.c. at 30 μl per hind footpad. Disease symptoms and mortality were recorded daily. Most mortality occurred within 12 days after infection but observations continued until 28 days post-infection. LD_{50} of HSV in mice is highly dependent on age, sex and ambient temperature of the animal house. The value was carefully titred for each set of experiments. Virus clearance was examined by infecting them s.c. with a sublethal dose of HSV-1 in the right pinna (Nash *et al.*, 1980) and virus infectivity remaining in the ear was determined by plaque assay using Vero cells, as previously described (Chan, 1989). Mice were also infected with a sublethal dose of virus s.c. in the footpad and base of tail for kinetic studies on the production of IL-3 by spleen cells or draining lymph node cells *in vitro*.

IL-3 production in vitro

Draining lymph nodes (DLN) or spleens were obtained from CBA mice at various days post-HSV-1 infection. Single-cell suspensions were dispensed at 5×10^6 cells per ml in RPMI-1640 medium without FCS but supplemented with 5×10^{-5} M 2-ME. Cultures with or without heat-inactivated (56°, 1 hr) virus antigen were incubated in an atmosphere of 5% CO_2 at 37° for 24 hr in 24-well Costar plates, and thereafter culture supernatant were collected and stored at −70°.

IL-3 assay

All assays were set up in triplicate in 96-well flat-bottomed tissue culture plates. The IL-3-dependent cell line 32D was washed three times with RPMI-1640 medium (Gibco, Grand Island, NY) and dispensed at 1×10^5 cells per well in 100 μl of RPMI-1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME. Then 100 μl of test culture supernatant were added and cultures were incubated at 37° and 5% CO_2 for 24 hr, pulsed for 18 hr with 1 μCi per well of [^{3}H]thymidine at 5 Ci/mmole (Amersham, Amersham, Bucks, U.K.) and harvested with a cell harvester. IL-3 activity was expressed as [^{3}H]thymidine uptake (c.p.m.). In preliminary experiments, it was established that there was essentially no difference whether IL-3 was assayed on 32DCL or on FCDP2, another IL-3-dependent cell line. IL-3 activity in all the supernatants tested decreased linearly with serial twofold dilutions of the supernatants.

Preparation and infection of mouse embryonic brain cells

Embryos were obtained from pregnant CBA mice killed at Day 17 of gestation. Heads were removed, washed three times with Dulbecco's modified Eagles medium (DMEM; Gibco) minced and transferred in 50 ml DMEM into a 250-ml fluted conical flask containing a small stirring bar and 5 ml of 0·25% trypsin. The flask was then incubated with stirring at 37° for 30 min. Trypsin digestion was stopped by adding 2 ml of FCS and the cell suspension filtered through sterile gauze, washed once in DMEM and resuspended in DMEM containing 10% FCS. The brain cell suspension was dispensed into a 24-well tissue culture plate at 5×10^5 cells per 0·5 ml. A further 0·5 ml of medium with or without appropriate amounts of cytokines and antibody was added to each well. The cells were incubated for 3 days at 37° in 5% CO_2, when another 0·5 ml of medium with or without cytokine and antibody was added and the cells incubated for a further 2 days before infection with 200 PFU of HSV per well. Infection was carried out by removing 1 ml of medium and addition of 0·1 ml virus (200 PFU). The virus was allowed to absorb for 2 hr and then 0·5 ml of medium with or without cytokine and antibody was added and the cells incubated for a further 48 hr before they were harvested by freezing. After another freeze-thaw cycle, the culture supernatants were assayed for virus production by the plaque assay on Vero cells. In some experiments, 1 ng/ml lipopolysaccharide (LPS; Difco Labs, Detroit, ME) with or without 1 μg/ml polymyxin B (PB; Sigma Chemical Co., Poole, Dorset, U.K.) was added as controls. Anti-IL-3 antiserum and control normal serum (final dilution 1/20) were mixed with 100 units purified mouse IL-3 (Genzyme, Kent, U.K.) before addition to the cultures. In preliminary experiments, the virus infecting dose was carefully titrated. The embryonic brain cells are heterogenous and highly susceptible to HSV infection and 200 PFU were found to be most practicable for providing a readout of 4–5 $\log_{10}$ of plaque.

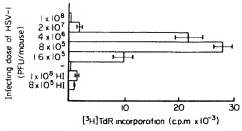

Figure 1. Effect of HSV infecting dose on IL-3 production by spleen cells of CBA mice. Mice were infected in the footpad with graded doses of HSV-1 or heat-inactivated (HI) HSV-1. Spleen cells were harvested 6 days later and stimulated for 24 hr with 7×10^6 PFU of HI HSV-1. IL-3 activity in the culture supernatants was measured as described in the Materials and Methods. Vertical bars = 1 SEM, $n = 3$.

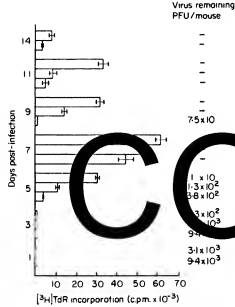

Figure 2. HSV titre and IL-3 activity in individual CBA mice at various times after sublethal infection in the pinna with 3×10^5 PFU of HSV-1. Vertical bars = 1 SEM of triplicate cultures.

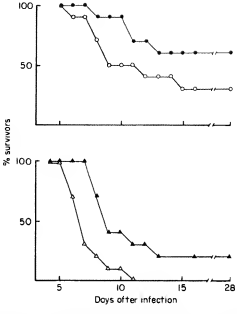

Figure 3. Effects of anti-IL-3 antibody on HSV infection in CBA mice. Groups of 10 mice were infected with $5 \times LD_{50}$ of HSV-1 and injected i.p. with: (a) monoclonal anti-IL-3 antibody (2E11, ○) or a control, non-cross-reacting anti-Rubella antibody (WRU, ●); or (b) polyclonal sheep anti-IL-3 antibody (△) or normal sheep immunoglobulin (▲). The results are representative of four similar experiments. The value for a [illegible] statistic on 1 degree of freedom is 3·84 for the 5% level and 6·63 for the 1% level. Thus, there is a significant difference in the average death rate between the treated group and the control group at the 5% level.

The amounts of IL-3 and anti-IL-3 were also carefully titrated in preliminary experiments to obtain a practicable level of neutralization *in vitro*.

Statistical analysis

All experiments were repeated two to three times. Significance was analysed by Student's *t*-test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Effects of HSV infecting dose on IL-3 production *in vitro*

Results in Fig. 1 show that the highest level of IL-3 was produced *in vitro* by spleen cells from mice infected with 8×10^5 PFU per mouse of HSV-1. This is a sublethal dose of virus for the age (11-week-old) of the mice used. Little or no IL-3 was produced when lethal doses of virus were used (2×10^7 PFU per mouse). At this dose mice start to die from Day 7. Interestingly, IL-3 production is primed by virus infection but not by inactivated virus. Heat-inactivated HSV (HI-HSV) at the optimal infecting dose of 8×10^5 PFU induced only very low IL-3 production. Even a 100-fold increase of inactivated virus (to 1×10^8 PFU) did not enhance the IL-3 response (Fig. 1).

Kinetics of *in vitro* IL-3 production

Since IL-3 production by spleen or DLN cells *in vitro* requires infection, we therefore investigated whether the level of IL-3 production varies with the degree of virus clearance from the primary site of infection. While the mice used in Fig. 1 were infected s.c. in the hind footpads, it is experimentally more convenient to monitor virus clearance in the pinna. Figure 2 shows the results comparing virus titre in the ear and IL-3 production by the spleen cells for individual mice over a period of 14 days post-infection. When mice were sublethally infected in the pinna, virus infectivity reached a peak by about Day 4, and was quickly cleared and became undetectable in the pinna by Day 7–9. Virus was cleared from the right pinna of all disease-free mice by Day 7 post-infection. IL-3 was not detectable up to Day 3 post-infection. The level of IL-3 production increased from Day 5 and reached a peak on Day 7 and slowly fell off to low but still significant levels by Day 14. Spleen cells from the mouse with clinical signs of viral encephalitis and with 75 PFU of virus remaining in the ear on Day 9,

Table 1. Effects of IL-3 on HSV-infection of embryo head cells of CBA mice *in vitro**

Material added†	HSV-1 (log_{10} PFU/ml)	P‡
Exp. 1		
Medium	3·98 ± 0·32	—
WEHI-3B	1·96 ± 0·96	< 0·05
IL-3	< 1	< 0·001
Exp. 2		
Medium	3·80 ± 0·25	—
LPS	4·33 ± 0·21	NS
Medium + PB	3·64 ± 0·68	NS
LPS + PB	3·82 ± 0·34	NS
IL-3	1·63 ± 0·45	< 0·01
IL-3 + PB	1·88 ± 1·03	< 0·05
Exp. 3		
Medium	4·53 ± 0·50	—
IL-3 + normal serum	1·85 ± 0·74	< 0·005
IL-3 + anti-IL-3	3·40 ± 0·72	NS
IL-3 + anti-IFN-α, β	5·09 ± 0·21	NS
IL-3 + anti-IFN-γ	4·52 ± 0·10	NS

* For details see the Materials and Methods.

† LPS (1 ng/ml) was used as control for possible contamination of this material with the IL-3 preparation. Polymyxin B (PB) (10 μg/ml) [illegible] inhibition of LPS. Data for IL-3 + normal serum [illegible] pooled results for all control normal sera (anti-IL-3, anti-IFN-α, β and γ).

‡ *P* values compared to medium control; NS = not significant. Each experiment was performed twice with similar results.

produced insignificant amounts of IL-3. Spleen cells from all other mice with clinical signs of viral encephalitis consistently produced little or no IL-3 *in vitro* (data not shown). Thus the overall picture suggests that the production of IL-3 by spleen cells correlates with the onset of virus clearance in HSV-1-infected CBA mice.

Effects of anti-IL-3 antibody on HSV infection in mice

Depending on the age of mice and dose of infecting virus, the kinetics of disease pathogenesis following a lethal s.c. infection are clinically evident by varying degrees of paraplegia, immobility, hunching and incontinence on Days 7–9, with death ensuing in the majority of cases on Days 8–12. Some mice do recover from mild paraplegia. Since increasing levels of IL-3 are produced in mice at the onset of recovery (Fig. 2), anti-IL-3 antibody was administered to the mice at Days 0, 1, 3 and 5 post-infection to determine if the antibody can influence disease development. In a series of experiments it was evident that mice receiving anti-IL-3 monoclonal antibody (2E11) developed exacerbated disease compared to control mice receiving an irrelevant antibody (WRU) (Fig. 3a). Similar results were obtained with the sheep anti-IL-3 antibody (Fig. 3b). The anti-IL-3 antibody-treated mice developed disease symptoms earlier, starting at Day 5, with mice dying from Day 6 instead of Day 8 in the controls. Mortality at the end of 28-day period was twice as many as in the control group.

Effects of IL-3 on HSV-1 infection of embryo brain cells *in vitro*

Since the pathology of the disease lies in the central nervous system, embryo brain cells of CBA mice were used to examine the *in vitro* effect of IL-3 as a regulator of pluripotent progenitor stem cells. IL-3 may act directly on the development of microglial or indirectly on HSV-1 replication in neuronal and glial cells. Primary mouse embryonic head cells grown in the presence of murine IL-3 show a 1000-fold decrease in virus titre compared with untreated control cells or cells treated with LPS ± PB (Table 1). The lack of effect of deliberately added LPS shows that the decrease in virus titre is not due to contaminating concentrations of LPS in the IL-3 preparation. This is confirmed by the presence of PB (an inhibitor of LPS), which has no effect on the activity of the IL-3 used. When anti-IL-3 antiserum was mixed with IL-3 before addition to the cultures, the inhibition of virus replication was reversed compared with cultures treated with normal serum. The inhibitory effect of IL-3 can also be reversed by anti-IFN-α, β and anti-IFN-γ antibody (Table 1, Exp. 3). The anti-IFN antibodies had no effect on IL-3 activity when assayed on an IL-3 dependent cell line (data not shown).

DISCUSSION

In previous experiments it has been shown that during acute HSV-1 infection the neuropathology induced in the brain includes [illegible] and accumulation of macrophages and amoeboid microglia in brain parenchyma and also as perivascular infiltrates that express the CD4 antigen (Chan, Cavanagh & Luk, 1989). The change in microglial morphology from ramified to amoeboid and the coincidental increase in CD4 also evident after inflammation and changes in the blood–brain barrier (Perry & Gordon, 1987). Therefore the accumulation of macrophages following HSV infection of brain cells may be due to an invasion and proliferation of blood-derived monocytes and/or activation and proliferation of microglial cells, induced perhaps by an influx of immunomodulators like the colony-stimulating factors. Since IL-3 can act as a growth factor for microglial cells (Yung *et al.*, 1981), this led to the question of whether IL-3 protects against HSV-1 infection via its effect on microglial cells.

While macrophages have long been implicated in the inhibition of virus replication and hence resistance to HSV infection in mice (Johnson, 1964), it was only recently that cytokines were shown to be involved. Using the mouse model of intraperitoneal (i.p.) infection, Engler *et al.* (1981) demonstrated that there is a marked increase of IFN (α, β, γ) production with resultant increase in natural killer (NK) cell activity in peritoneal exudate cells from HSV-infected mice. In addition, Domke *et al.* (1985) demonstrated that IFN (α, β, γ) can directly inhibit virus replication at an early step prior to or during the synthesis of early viral proteins and that IFN-γ act synergistically with IFN-α or IFN-β. Recently, Kohl *et al.* (1989) showed that injection of human recombinant IL-2 or IFN-γ protected neonatal mice from a lethal HSV infection. Since antibody to IFN-γ ablated protection induced by either of the two lymphokines, they postulated that IL-2 mediated protection by stimulating macrophage activity, via T-cell-produced IFN-γ. Since murine bone marrow-derived macrophages have been shown to release IFN when exposed to CSF-1 (Fleit & Rabinovitch, 1981; Moore *et al.*, 1984), it is conceivable

that microglia may release IFN when exposed to IL-3. This possibility is clearly supported by results shown in Table 1. However, the culture supernatant of head cells treated with IL-3 contained only low levels of IFN which were barely detectable by a radioimmunoassay or a cytopathic assay (data not shown). It may be that the amounts of IFN induced by IL-3 were low but effective against HSV in a microenvironment.

The priming of IL-3 production paralleled that of the onset of virus clearance from the peripheral site of infection. This in turn correlated with an increase in T-cell activity in response to HSV infection. It has been reported that astrocytes produce an IL-3-like activity (Frei *et al.*, 1986). However, this has not been demonstrated by molecular analysis or Northern blotting. T cells play a crucial role in protection against HSV (Nash *et al.*, 1987, 1980; Nagfuchi *et al.*, 1982; Larsen *et al.*, 1983; Nash & Gell, 1983; Sethi *et al.*, 1983) and, at present, the most likely source of IL-3 in the brain of HSV-infected mice appears to be specifically activated T cells. However, when mice were lethally infected (Fig. 1) or showed clinical signs of viral encephalitis (Figs 2 and 3), only low levels of IL-3 were produced *in vitro*. It appears that while live virus infection is required to induce IL-3 release by activated T lymphocytes (Fig. 1), a debilitating dose of virus on the other hand would have the opposite effect. The lack of IL-3 production in this case may result from an incapacitation of the immune response, since T lymphocytes which normally are not permissive to HSV replication may become infected on activation by IL-2 (Braun & Kirchner, 1986).

While administration of monoclonal anti-IL-3 antibody to mice infected with HSV exacerbated disease, suggesting that the presence of IL-3 can protect against HSV infection, the reverse effect was seen when mice infected with *Leishmania major* (Chan *et al.*, 1988) or *Plasmodium berghei* (Grau *et al.*, 1988) were simultaneously injected with IL-3 antibody. In the latter situation, together with anti-GM-CSF antibody. In these cases, IL-3 appears to exacerbate disease development. Thus IL-3 has pleiotropic effects and, depending on the pathogenesis of a disease, IL-3 can either protect or exacerbate.

Though the evidence obtained with the administration of anti-IL-3 antibody is indirect, we did not inject IL-3 into mice *in vivo* because of its short half-life and because the use of diffusion chambers only produces a localized concentration. Therefore IL-3 was added to head cell cultures to study its effect *in vitro*.

Head cell cultures containing a heterogeneous population of cells were used to simulate the *in vivo* situation. Addition of IL-3 to the cultures reduced the virus yield by 10^3 PFU per ml compared with control cultures grown in the medium alone or in the presence of LPS or PB. Thus the reduced virus yield is not due to the mitogenic effect of contaminating concentrations of LPS in the IL-3 preparation used. Addition of anti-IL-3 antibody, on the other hand, reversed the effect of IL-3. Furthermore, the inhibition of HSV replication by IL-3 was also completely abolished by anti-interferon antibodies which have no direct effect on IL-3 activity. Our results therefore demonstrate that IL-3 plays a protective role against HSV-1 infection in the mouse. It does so probably by acting on brain cells to produce other cytokines such as IFN-α, β and γ, which then inhibit virus replication. The identity of the brain cells involved in the present system is unclear. It may require the participation of a number of different cell types, some of which respond directly to IL-3. Fibroblasts and lymphocytes appear not to be the targets of IL-3, since IL-3 receptors have only been found on cells of the monocytic lineage (Ziltener *et al.*, 1988). The detailed mechanism of IL-3-mediated inhibition of HSV replication, as well as the cell types involved is currently under investigation.

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